

Effect of Light and Carbon Source for the Production of Lipid by *Chlorella pyrenoidosa* using Agricultural Wastewater in Malaysia

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Abstract

The light regimes submitted to the algal cultures are considered to be an important factor in determining the productivity and yield of photosynthetic reactions. The aim of this study was to evaluate the effect of photoperiod on the production of biomass and lipid by locally isolated microalgae, *Chlorella pyrenoidosa* (*C. pyrenoidosa*). The cultures were grown at temperatures of 24°C - 26°C and supplied with a light density of 150 mol m⁻²s⁻¹. The light cycles evaluated were 24:0, 16:8, and 8:16 (hr in light: hr in dark) respectively. The highest lipid content achieved 18 mg lipid/mg cell dry weight (CDW) when the *C. pyrenoidosa* was cultured under continuous light condition (24 hr); followed by 16:8 and 8:16 with value of 13.10 and 9.43 mg lipid/mg CDW, respectively. It was shown that POME could be used to grow *C. pyrenoidosa* where the corresponding specific growth rate was reasonably well compared to the glucose as the growing medium. The production of *C. pyrenoidosa* was optimized for biomass production using a Hybrid Photo Bioreactor (HPBR) in which comprised of a 5L batch culture over 14 days. It produced a maximum biomass production of 0.68 g L⁻¹·d⁻¹·mg CDW⁻¹. Prior to this, the inoculant was grown in a 250 mL flask culture for 28 days which produced a maximum biomass of 0.009 g L⁻¹·d⁻¹·mg CDW⁻¹. After two days, both organic carbon and nutrients decreased gradually as the carbon source was used for the microalgae growth and production of lipid. By the end of this experiments, 34%, 65%, and 47% of organic carbon was removed by *C. pyrenoidosa* at carbon per total nitrogen (C/TN) ratio of 100:7, 100:13, and 100:32 respectively. The results showed that in the presence of light, *C. pyrenoidosa* contributed more significantly to the reduction of organic and inorganic compounds when glucose was used as the carbon source compared to POME.

Keywords: *Chlorella pyrenoidosa*, lipid productivity, photoperiod, carbon source, palm oil mill effluent (POME)

1. Introduction

Microalgae are a eukaryotic cell that consists of organelles, including nuclei and plastids. Proteins, carbohydrates, and lipid (natural oil) are included in the biomass. The percentage of chemical composition varies with the type of algae (Kamyab et al., 2013). In fact, microalgae have become the focus of attention for both wastewater treatment and biomass production as early as 1950s (Li, 2008). There was much interest in the development of integrated algae oil and the wastewater treatment process (Kamyab et al., 2014a).

Microalgae capture light energy and the energy is used for cell synthesis and convert the inorganic molecule into simpler sugar and act as the source of energy for cell metabolism. While the mechanism of photosynthesis in

microalgae is similar to that of higher plants, they are generally more efficient converters of solar energy because of their simple cellular structure (Khan et al., 2009). In contrast, many polar lipids are associated with various types of membranes and are likely to dominate the total lipid pool when the light is limited given that the nutrients are sufficient to support rapid growth (Kamyab et al., 2013).

Furthermore, photoautotrophic (Chen et al, 2011; Mata et al, 2010), heterotrophic (Chen et al, 2011; Huang et al., 2010; Liang, 2009; Xu, 2006; Wen and Chen, 2000; Miao and Wu, 2006; Shen, 2010), and mixotrophic (Chen et al., 2011) are various microalgae cultivation methods that have been reported. Moreover, various parameters such as light, temperature, pH, salinity, irradiance, and most markedly, the nutrient availability such as carbon (Pulz et al., 2008; Lee and Lee, 2001; Rodolfi et al., 2009; Rubin, 2008; Costa and de Morais, 2011; Yue and Chen, 2005; Wu et al., 2009), nitrogen (Martin-Jézéquel et al., 2000; Perez-Garcia et al., 2011), phosphorus (Niklas, 2008; Martínez, 1999), and metal elements (Zhang et al., 2002; Roden and Zachara, 1996; Raven et al, 1999) have been shown to affect both the lipids composition and the content in many microalgae (Rodolfi et al., 2009).

Palm oil mill effluent (POME), a wastewater rich in organic content, is a promising substrate in the scenario of algae bloom. It is desirable to enhance further the lipid content within the algae to be further used in the biofuel industry (Kamyab et al., 2014a). In addition, palm oil industry is growing rapidly among the potential tropical countries especially in Malaysia. Although POME contains low level of degradable organic matter (3-5%), discharge activities of improperly treated POME in a large volume could create adverse impact on the environment. POME is a thick brownish viscous liquid waste and non-toxic as no chemicals are added during the oil extraction but it has an unpleasant odor. It is predominantly organic in nature and highly polluting due to the discharge in large volumes (Kamyab et al., 2014b; Ahmad et al., 2003; Hassan et al., 1999).

Some microalgae are most productive at high temperatures and bright light, while full sunlight could retard the growth of other microalgae. Due to the competition of faster growing microalgae, certain microalgae species cannot be grown reliably and constantly (Sheehan et al., 1998).

The key limiting factor for the growth of microalgae is the light intensity. Consequently, a suitable light intensity for microalgae cultivation needs about 1/10 of the amount of direct sunlight. This photosynthetic microorganism produces microalgae biomass by utilizing the sunlight, water, and carbon dioxide. About 25% of the biomass produced during daylight might be consumed during the night to sustain the cells until sunrise (Chisti, 2007; Kamyab et al., 2014c).

The yield and production of fatty acids in the photoautotrophic systems are low, this is due to the insufficiency of light caused by mutual shading of cells (Chen et al., 2011). In order to enhance the production of fatty acid by microalgae, the development of a heterotrophic growth system is required (Wen and Chen, 2000).

From a technological point of view, it would be very interesting if the microalgae are capable to store a considerable amount of light energy to sustain their growth in the dark. However, as the light energy is absorbed by photosynthetic pigments in microalgae, only a small fraction of the energy was stored. Over 60% of the fraction dissipated in the form of heat energy during the reactions and limited capacity of the microalgae to store the light energy account for the loss energy received from the light (Janssen et al., 1999; Suggett et al., 2010).

Studies have also shown that both the light cycle and mixing turbulence influenced the photosynthetic activity as well as the growth rates of microalgae in a photobioreactor (Janssen et al., 1999, 2000). The availability of light is a limiting substrate in these systems, which are affected by the light/dark zones that depend mainly on the configuration, stirring, and mixing in the reactor, as well as the possibility of culturing with discontinuous periods of light energy supply and temperature (Grobbelaar, 1994; 2009). The shortage was overcome by increasing the turbulence or mixing intensity to increase the productivity and photosynthesis efficiency. Both light/dark cycle and turbulence are two separate components, but they function synergistically (Grobbelaar, 1994). The design of a

photobioreactor such as low-cost design and flat panel airlift (FPA) is equally important to obtain high biomass productivity (Lehr and Posten, 2009; Stephan et al., 2002). In addition, the availability of light in a typical photobioreactor is another factor that determines the cell densities. High cell densities account for mutual shadings and limit the light intensity within the reactor (Evers, 1991; Grobbelaar, 2006). This study focuses on the effect of light/dark on the growth of microalgae while the effect of mixing intensity was not the scope of this study.

According to most authors, when cells are exposed to different light intensities, with a considerable effect on the system performance, it was found that the variations in both the growth rates and maximum cell density as a function of the duration of the light cycle showed that the cell concentration decreased proportionally with the fraction of time that the microalgae were exposed to intermittent light conditions compared to continuous illumination. Higher specific light absorption together with the lower specific growth rate most probably contribute to the reduction of the biomass production of *C. pyrenoidosa* under intermittent illumination. A similar study conducted by Janssen et al. (1999) revealed that *C. reinhardtii* increased its specific light absorption by increasing the content of chlorophyll a under light/dark cycles. The amount of chlorophyll a content was doubled under the intermittent illumination in comparison to continuous illumination. In short, whether the continuous illumination is beneficial to promote the growth of biomass and/or chlorophyll a, it could be varied based on specific strain of microalgae and possibly the combination of operating conditions.

The present study has successfully validated *C. pyrenoidosa* as viable microalgae strain to use POME as a growth medium to produce reasonable lipid content. In other words, the carbon sources in POME can be consumed as an alternative carbon source. In addition, a biological process was developed in order to enhance the production of lipid from microalgae in POME. The *C. pyrenoidosa* used for this study was screened and isolated from the dominant strains of microalgae collected from the POME. A range of operational parameters, i.e. carbon to nitrogen (C/N) ratio, photoperiod, and organic loading rate were investigated to study the maximized production of biomass and lipid in *C. pyrenoidosa* using HPBR as the reactor.

2. Materials and Methods

2.1 Culture medium and source of POME

C. pyrenoidosa was isolated from a POME pond located in Johor Palm Oil Mill, Kahang, Johor, Malaysia. The cultures were cultivated and maintained in a 10-fold diluted POME provided with cultural conditions of 24°C- 26°C, with photoperiod of 8h:16h (in Light: in Dark), pH ranging between 6.5-7.5, and light intensity of 150 mol m² s⁻¹. POME was diluted 10 times to reduce the shadings effect on the microalgae growth. The growth of *C. pyrenoidosa* was assessed by optical density (OD) at a wavelength of 600 nm, chlorophyll content.

2.2 Laboratory scale of photo cultivation system

The laboratory scales of experiments were conducted in a 250 mL Erlenmeyer flasks. The cultures were added with a specific range of POME after dilution. Dissolved oxygen (DO), pH, and temperature were monitored every day. The cultures were maintained under a fluorescent lamp with a light intensity of 22.25 μmol m⁻² S⁻¹ for 16 h of light: 8 h of dark period which were set-up via relay controller for all experiments that were carried out in this study. The pH of the medium was 7 and the temperature was 27°C which was the room temperature with manual swirling applied once a day.

2.3 Kinetic parameters

The specific growth rate (μ) was determined by Equation (1):

$$\mu = 1/t \ln (X_m/X_0)$$

(1)

where: X_m = concentration of biomass at the end of a batch run
 X_0 = concentration of biomass at the beginning of a batch run,
 t = duration of the batch run (day)

The biomass productivity (P_{X/X_0}) was calculated by Equation (2):

$$P_{X/X_0} = (X-X_0) \cdot t^{-1}$$

(2)

where: X = concentration of biomass at the end of a batch run
 X_0 = concentration of biomass at the beginning of a batch run,
 t = duration of the batch run (day)

The productivity of lipid (P_{lipid}) was calculated by Equation (3):

$$P_{lipid} = Cl/t$$

(3)

where: Cl = concentration of lipids at the end of a batch run
 t = duration of the run (day)

2.4 Chlorophyll a content

Eight mL of microalgae cells was harvested through centrifugation at 5000 rpm for 5 minutes. The pellet was placed in a sonicator for 1 minute and resuspended in 1 mL of a 90% aqueous solution of acetone. Then, the pellet was mixed with 3 mL of aqueous acetone and left in a dark room at 4 °C for at least 2 h. Later, the pellet was separated by centrifugation at 5000 rpm for 5 minutes. Finally, the pellet was transferred into cuvette bottles to prepare for the measurements. All analyzes followed the standard method 10200 H (Eaton et al., 2005). The content of chlorophyll a was calculated based on Equation (4).

$$\text{Chlorophyll } a \text{ (mg / L)} = \frac{26.7 (664_b - 665_a) \times V_1}{V_2 \times L}$$

(4)

where V_1 is the volume of the extract (L), V_2 is the volume of sample (m^3), L is the width of the cuvette (cm), and 664_b and 665_a are the optical densities of the 90% acetone extract before and after the acidification, respectively.

2.5 Quantification of biomass and proximate analyzes of biomass components

To determine the biomass of microalgae, 4.7 cm Whatman GF/C glass fiber filters were dried at 90 °C for 4h and then placed in a desiccator to cool to room temperature and weighed. Then the biomass was determined by filtering 10mL of the culture and passed through the pre-weighed filters, dried, and weighed as mentioned above. The remains of *C. pyrenoidosa* cells were assayed based on the standard methods (Griffiths and Harrison, 2009).

2.6 The measurement of lipid content-Nile Red (NR) method

A fluorescence dye NR was used to stain the lipid found in the culture. The quantification of the cellular neutral lipid was carried out with a fluorescent spectrophotometer (Hitachi F-4500) (Griffiths and Harrison, 2009; Kamyab et al., 2013). The cultures were mixed with NR solution, stored in 0.1mg.ml⁻¹ of acetone for 7 minutes before analyzing. The relative fluorescent of NR for lipid was obtained after the subtraction of autofluorescent of algal cells and self-fluorescent of NR from the gross reading at a wavelength of 580nm.

3. Results and Discussion

3.1 Effect of photoperiod on the production of biomass and lipid by *Chlorella Pyrenoidosa* in POME

The specific growth rate of *C. pyrenoidosa* under different photoperiod is presented in Figure 1. The maximum amounts of lipid content achieved at the end of cultivation at three different ratios of light to dark were determined. There was a great difference in the maximum cell concentrations achieved under continuous light condition (24:0) as compared to the one under intermittent light condition, namely 16:8 and 8:16 (light:dark) period. Among the three light and dark cycles, the highest lipid content was achieved at 17.00 mg/mg CDW when the *C. pyrenoidosa* was cultured under continuous light condition (24:0), then followed by (16:8) and (8:16) with the value of 13.10, 9.43 mg/mg CDW, respectively. It clearly showed that the photo duration significantly influenced the lipid content as well as the cell growth rates.

Under these light regimes, *C. pyrenoidosa* is unable to store light energy during the light period to sustain its growth during the dark period at the same rate as under continuous illumination. It was suspected that the loss of biomass under dark/light cycle was most probably due to the photorespiration (Chisti, 2007; Lee and Lee, 2009).

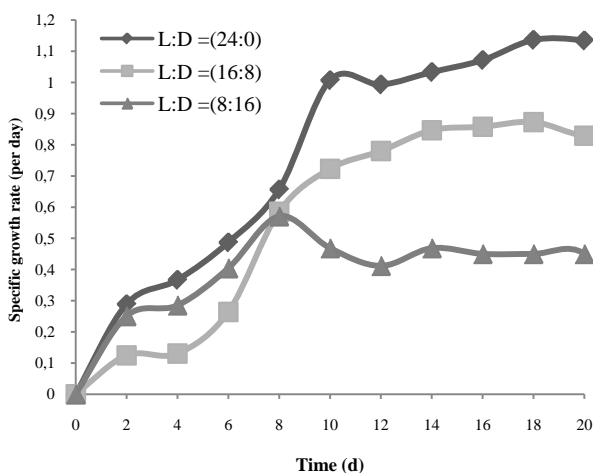


Figure 1: The effect of light and dark cycles on the specific growth rate of *C. pyrenoidosa*

3.2 Production of biomass and lipid by *Chlorella pyrenoidosa* in HPBR

In order to determine the feasibility of larger scale cultivation, *C. pyrenoidosa* was grown in a lab scale hybrid photobioreactor to examine whether a higher biomass concentration and lipid content could be reached. The process was divided into two sequential stages: flask scale (250 mL) followed by lab scaled bioreactor (5 L) as shown in Figure 2.

In order to maximize the production of the culture over a short period of time, *C. pyrenoidosa* were cultured using batch mode. With a batch culture, the culture was started at low density and supplied with sufficient nutrients that enabled the culture to proliferate to their maximum density. Hence, it enhanced the content of lipid. The density achieved was greater than that achieved through continuous culture as it was only maintained for a short period of time before the next culture (in HPBR) was started. Batch mode cultivation allowed the harvest of microalgae at their peak density, and continuously produced higher cell density before the next cultivation.

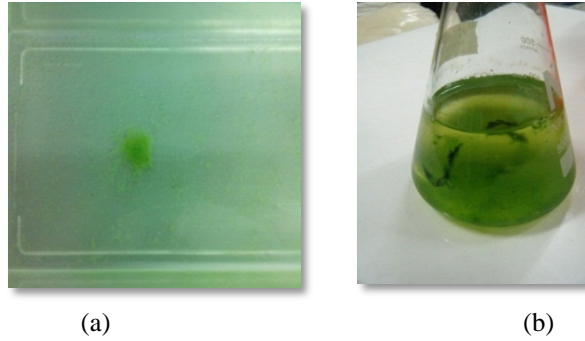


Figure 2: (a), (b) *C. pyrenoidosa* cultivation in batch modes. The green color clearly indicated the growth of *C. pyrenoidosa*

Cultivation in HPBR was performed in a 5L stirred tank reactor with turbine impellers. The double vessel has an inner diameter of 145mm and inner height of 300mm. On the other hand, the 250 mL Pyrex Erlenmeyer flasks were used for the experiments in flask cultures. The production of *C. pyrenoidosa* was optimized for the biomass production with HPBR in a 5L batch culture over 14 days, producing a maximum of $0.68 \text{ g L}^{-1} \text{ d}^{-1}$ CDW; 250 mL flask culture prior to optimization produced $0.009 \text{ g/L}\cdot\text{d}$ maximum biomass.

Light intensity plays a major role in the productivity of microalgae culture (Grobbelaar et al., 1996). At higher depths and cell concentrations the light intensity must be increased to penetrate through the culture (e.g. 1000 lux is suitable for Erlenmeyer flasks, 5000-10000 is required for larger volumes). Hence, flask scale cultivation has a shorter optical path compared to HPBR. Therefore, it increased the volumetric productivity. However, the result obtained clearly indicated lower biomass productivity compared to that in a reactor scale. Results in Table 1 shows that the growth of microalgae was offset by the volume of the flask.

Mixing the culture manually or by aeration is essential to suspend the biomass and to enhance the interaction between a liquid nutrient and the cells. Mixing will also prevent gradients of nutrients, pH, and the temperature inside the reactor. Mostly, aeration is used for mixing. In that case, shear stress is avoided. At the same time, maintaining the pH and temperature at optimal temperature are rather easy. A reactor scale, especially the commercial scale, controlling temperature is expensive. Table 1 summarizes the comparison between flask scale and HPBR in terms of physical operation, biomass productivity, and the content and productivity of lipid.

Table 1: Comparison between flask scale and HPBR in terms of physical operation, biomass productivity, and the content and productivity of lipid.		
	Flask culture	HPBR
Average Biomass productivity (mg/L·d)	9.5	130
Average lipid content (% of biomass)	21	-
Average Lipid productivity (mg/L·d)	1.78	230
Type of operation	Manual	Semi- auto
Volume of reactor (L)	0.25	5
Dimension (mm x mm)	132 × 82	300 × 145
Mixing	Easier	Difficult
Light path	Shorter	Deeper, may reduce light availability to the culture

3.4 Organic carbon substrate and nutrient utilization rate via *C. pyrenoidosa* from settled raw palm oil mill effluent (POME) for lipid production

Figure 3 shows the rate for organic carbon and nutrient utilization with the operation time for three different C:TN ratio (100:6; 100:17; 100:32). *C. pyrenoidosa* showed a general trend of short adaptation phase towards the presence of both organic and inorganic compounds in the wastewater. After two days, both organic carbon and nutrients started to decrease gradually, assuming that the microalgae used carbon source not only for growth but also for lipid production.

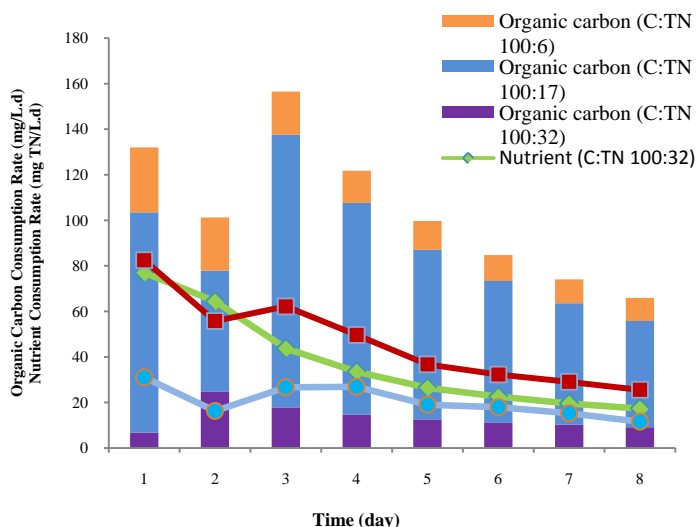


Figure 3: Utilization rate of nutrients and organic carbon substrate rate during the experiment

By the end of the experiment 34%, 65%, and 47% of organic carbon was removed by *C. pyrenoidosa* for the ratio of 100:7; 100:13, and 100:32 respectively. It means that the algae species could utilize different organic compounds as carbon sources (Li et al., 2008). Further studies were carried out to determine the lipid content and its productivity in *C. pyrenoidosa*.

3.5 Comparison of MLVSS/MLSS ratio and lipid content between POME and $C_6H_{12}O_6$ in batch cultures

Lipid productivity in the microalgae cells was 0.085 mg/mg CDW·day with POME feeding, and 0.136 mg lipid/mg CDW·day with $C_6H_{12}O_6$ feeding, which was about one and half times less than POME as carbon source. The $C_6H_{12}O_6$ is most suitable for microalgae growth because $C_6H_{12}O_6$ promoted physiological changes in microalgae, which strongly affects the metabolic pathways of carbon assimilation size of the cells, volume densities of storage materials such as starch, lipids grains and protein, chlorophyll, and vitamin content (Perez-Garcia et al., 2011). Meanwhile, POME contains various organic and inorganic compounds such as N, Fe, Zn, P, Mg, Ca, and K which require a fix concentration for the microalgae to grow effectively (Lee and Lee, 2001).

The ratio of MLVSS/MLSS can detect the quantity of lipid content from microalgae, but not for specific value. The comparison between MLVSS/MLSS ratio and the lipid content using POME and $C_6H_{12}O_6$ by *C. pyrenoidosa* performance is presented in Figure 4. Highest lipid content was achieved by $C_6H_{12}O_6$ as the substrate with the value 3.06 mg lipid/mg CDW, while POME has a value of 2.16 mg lipid/mg CDW. The correlation of MLVSS/MLSS ratio and lipid content was indicated by POME as a substrate, meaning that the lipid content increased by increasing the MLVSS/MLSS ratio. This occurs may be because the *C. pyrenoidosa* consumes substrates has affected both of

growth and storage lipid. In fact $C_6H_{12}O_6$ is more suitable substrate than POME for *C. pyrenoidosa* growth and for lipid generation. Furthermore, the maximum value between

POME and $C_6H_{12}O_6$ in terms of specific growth rate, carbon source consumption rate, chlorophyll productivity, biomass productivity, and MLVSS/MLSS ratio as shown in Table 2.

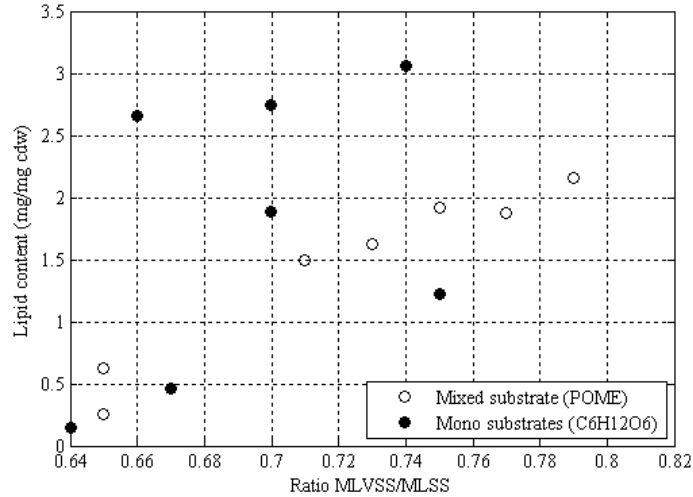


Figure 4 Comparison of MLVSS/MLSS ratio and lipid content between POME and $C_6H_{12}O_6$ in batch cultures

Table 2: Maximum value for specific growth rate, carbon source consumption rate, chlorophyll productivity, biomass productivity, and ratio MLVSS/MLSS

Parameter	Unit	Carbon source	
		mixed substrate POME	mono-substrate ($C_6H_{12}O_6$)
max specific growth rate (μ_m)	per hour	0.007	0.008
max carbon source consumption rate	mg/L·day	32.50	18.75
max chlorophyll II productivity	mg/L·day	0.290	0.262
max biomass productivity	mg/L·day	100	70
max lipid productivity	mg lipid/mg CDW·day	0.310	0.230
max ratio MLVSS/MLSS	-	0.79	0.75

4. Conclusion

The highest lipid content was achieved when the *C. pyrenoidosa* was cultured under continuous light condition (24 hr) at 18.00 mg lipid/mg CDW, and then followed by 16:8 and 8:16 with value of 13.10, 9.43 mg lipid/mg CDW, respectively. Lipid productivity in the microalgae cells was 0.085 mg/mg CDW·day with POME feeding, and 0.136 mg lipid/mg CDW·day with $C_6H_{12}O_6$ feeding, which was about one and half times less than POME as carbon source. The $C_6H_{12}O_6$ is the most suitable for microalgae growth because $C_6H_{12}O_6$ promotes physiological changes in microalgae.

The comparison of carbon sources between $C_6H_{12}O_6$ and POME were applied for the best growth microalgae strains. The result showed that *C. pyrenoidosa* significantly contributed in $C_6H_{12}O_6$ rather than POME as carbon sources. The maximum growth rate (μ_{max}) at the end of experiments was monitored at 340 mg MLSS/L with $C_6H_{12}O_6$ and 0.290 mg MLSS/L with POME substrates. Furthermore, lipid productivity achieved by $C_6H_{12}O_6$ at

was approximately one half less than POME as the carbon source. The development of technologies to optimize the microalgae production, oil extraction, and biomass processing has the capacity to make significant contributions towards this goal. Highest lipid content achieved by C₆H₁₂O₆ as a substrate was 3.06 mg lipid/mg CDW, while POME has a value of 2.16 mg lipid/mg CDW.

The proper duration of illumination is essential for both growth and lipid productivity of *C. pyrenoidosa*. The microalgae achieved relatively high biomass productivity of 3 g/L·d and high lipid productivity of 1.3 g/L·d under continuous illumination.

The comparison of *C. pyrenoidosa* cultivation in flask and HPPBR scale clearly showed higher productivity for both lipid productivity and biomass productivity of *Chlorella pyrenoidosa* in diluted POME using HPBR. Flask is a promising vessel which provides a shorter light path, hence enable higher growth of microalgae. Yet in this study, the production of both lipid and biomass was restricted by the volume of the vessel used. In conclusion, *C. pyrenoidosa* was found to produce highest lipid when it was grown under C:TN ratio of 100:6, under continuous illumination, and with OLR of 36 kg COD/m³·d using HPBR.

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